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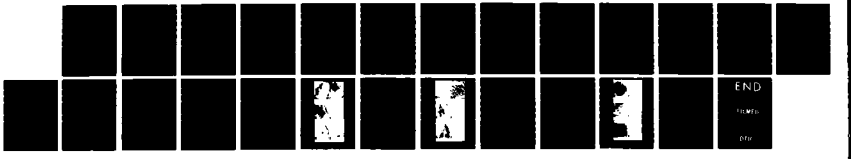

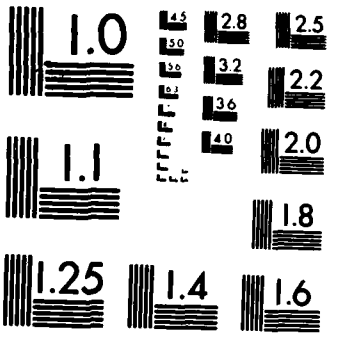

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TISSUE CULTURE IN VIVO MODELLING OF CORNEAL OPACIFICATION AND OCULAR INJURIES BY PULSED MILLIMETER WAVES

Annual Report

Dr. John R. Trevithick

June, 1983

June, 1982 - May, 1983

Supported by

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20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

The eventual aims of these experiments are to use intact corneas incubated in vitro and corneas of rats or rabbits exposed in vivo to high energy pulsed millimeter waves to study the development of corneal damage. Such experiments are expected (1) to establish conditions for corneal damage and (2) to elucidate the mechanisms by which the damage occurs.

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SUMMARY

In previous experiments progressive cellular damage occurred when cultured corneas were incubated after exposure to elevated temperatures. Following these experiments, this year immediate fixation was performed. Following this, the effect of a slightly different nonionizing radiation (ultraviolet) on cultured cornea was tested. Progressive damage, increasing with time of exposure to ultraviolet irradiation, involved loss of microvilli, some holes in cell surfaces and eventually lifting off of cells, leaving large areas of the corneal surface denuded of cells.

In vivo, preliminary experiments in which rabbit eyes were irradiated with millimeter waves have given results consistent with similar progressive damage to that found in vitro, with early damage evident as holes and pitting in cell surfaces, and eventually resulting in cell death and exfoliation of both endothelial and epithelial cells, leaving cell debris.

The severity of damage in these preliminary experiments appeared to be correlated with two factors: (1) peak power per pulse and (2) rate of increase of temperature. Even though only over 10 sec., the damage in eye used for SAR determinations was considerably more than that approximately 1 half the dose given over a longer period (20 min).

FOREWORD

A. List of Professional Personnel Employed on This Project

Principal Investigator	Dr. John R. Trevithick, Ph.D.
Research Associate	Dr. Margaret O. Creighton, Ph.D.
	Dr. Peter Galsworthy, Ph.D
Technician (Part-time)	Mrs. Andrea Hanington

B. Animal Care

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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Figure 2 Scanning electron microscopy of corneal epithelium: at short times after exposure to millimeter waves in vivo.

Figure 3 Scanning electron microscopy of corneal endothelium from short times up to long periods after exposure to pulsed millimter waves in vivo.

INTRODUCTION

Although millimeter wave radars are now strategically important, only one study of the effect of millimeter waves on the cornea has been reported (Rosenthal et al, 1975). This study did not use the pulsed mode of millimeter waves which is commonly in use in such radars. Because we have discovered apparent differences between similar doses of pulsed and CW microwaves (Stewart-DeHaan et al, 1980) in preliminary experiments and because we have succeeded in separating the effect of heating from the effects due to the electromagnetic field, for microwaves, we wished to devise a similar system for irradiation of the cornea in vitro which would offer similar advantages for the study of millimeter wave damage to the cornea.

The first step in these experiments was to devise appropriate media and conditions for the tissue culture of corneas, to be used for the experiments investigating their exposure to millimeter waves. The second step, also described in our Annual Report, June 1981 under DAMD17-80-G-9480, was to incubate the cultured corneas at different elevated temperatures in order to investigate the effect of incubation at elevated temperature on the cornea. The third stage, reported in our Annual Report, June 1982 under DAMD17-80-G-9480 and DAMD17-82-C-2018, was to test the effect of exposing incubated corneas to non-ionizing radiation, for which we selected ultraviolet as convenient and accessibile in our laboratory, since the appropriate millimeter wave irradiation apparatus was not yet operating. In 1983 the millimeter wave irradiation apparatus was functioning, permitting us to perform preliminary experiments on rabbits in vivo which are reported here.

MATERIALS AND METHODS

In Vivo Irradiation

New Zealand white male rabbits, average weight 3.6 kg were irradiated in a special rabbit carrier constructed of plexiglass and nylon parts which maintained their heads in a fixed position during the 20 min period of irradiation. The carrier was fastened to a large block of styrofoam by strong plastic lacing so that the optical axis of the rabbit's eye approximately coincided with the axis of the horn antenna emitting the millimeter waves. The distance from the cornea to the front of the antenna was measured. The power and interpulse interval was adjusted to compensate for distance when the rabbits were placed at various distances in order to change the peak power while maintaining the average power at the animal constant for exposures. For SAR determination the rabbit's corneal surface temperature was measured by recoding the temperature reading of an optical pyrometer during a 10 second exposure at the appropriate distance using an instrument power approximately 66 times that used for 20

min irradiations, which always resulted in a temperature elevation of no more than 43°c. The corneal temperature was allowed to return to 38°C or less prior to re-irradiating.

Staining of Corneas with Dyes to Detect Epitheilial Damage

In Vivo

Rabbit corneas were stained with 1% rose bengal in physiological saline or with sodium fluorescein dye prepared by placing a drop of saline on a fluorescein treated paper strip used for eye examinations, and touching this to the animal's eye. By manipulation of the eyelid, the drop of either dye was spread over the corneal surface prior to photography with visible light for rose bengal, or long-wave length ultraviolet, for fluorescein, using a 35 mm camera (Nikon) equipped with telephoto lens and extension tubes.

In Vitro

Rabbit eyes, obtained from a slaughterhouse, were stained with 1% sodium fluorescein in phosphate-buffered saline (PBS) after several different types of injurious treatment:

1. scratched with sharp tip of syringe needle
   or 5 minutes exposure to:

2. 5 m HCl
3. 1 m HCl
4. Acetone
5. 1 M NaOH
6. 0.1% Triton X-100

After three washes by dipping in PBS, fluorescein staining was observed by viewing under a long-wave ultraviolet lamp. Results are tabulated in Table 1.

Other Methods Reported in Previous Annual Reports

Phase contrast and interference phase microscopy, embedding in water-soluble media for thin sectioning, scanning electron microscopy, and various appropriate biochemical techniques were discussed in our 1982 annual report dated June 1982 for grant no. DAMD17-80-G-9480 and contract no. DAMD17-82-C-2018.

Results

Previous results reported in our Annual Reports, June 1981 under DAMD17-80-G-9480 and DAMD17-82-C-2018 included the development of tissue culture media which supported outgrowth of corneal epithelial cells observation of cytoskeleton by HVEM, and by a technique for removing the cell membrane by scotch "Magic" tape, and the progressive damage caused to incubated corneas both

by exposure to elevated temperature and exposure to ultraviolet light, resulting eventually in areas of exfoliation of epithelial and endothelial cells.

TABLE 1

Treatment:	Initial time, nothing seen
Nothing-control	Nothing seen
Scratches	Bright lines crisp +++++
5 N HCl - 5 mins	+++++
1 N HCl - 5 mins	++ slight
Acetone - 1 min	+++
1 N NaOH - 5 mins	+++++ gummy surface
Triton X100 - 0.1% - 5 mins	+++++

NEW RESULTS

(1) Fluorescein Staining of Corneas After Physical or Chemical Injury In Vitro

As illustrated in Table 1, staining with fluorescein was: (1) strong, in a line in area of corneas injured by scratching with a syringe needle; (2) strong, in area exposed to 6 m HCl for 5 min; (3) slight, in area exposed to 1 M HCl for 5 min; (4) moderate, in area exposed to acetone; (5) strong in area exposed to 1 M NaOH, (the surface appeared somewhat sticky); (6) strong in area exposed to Triton X-100 (0.1%).

(2) Further Studies of Elevated Temperature on Corneas In Vitro

The effect of elevated temperature was tested using two protocols: (1) in which a constant time of exposure was used and temperatures were varied from 35.5 to 50°C (see Table 2a) and (2) in which corneas were exposed at 39°C for times ranging from 0-45 min (Table 2b). These studies confirmed the previously observed progressive damage to the epithelium occurring with increasing temperature, and in the study of the time course of epithelial changes indicated a progressive disappearance of microvilli, which changed from small bumps becoming very long and thin and eventually disappearing after 45 min at 39°C. More extensive observations this time (Table 2a) indicated that endothelial damage could be detected in addition to the previously described progressive epithelial damage which led eventually to loss of epithelial cell microvillous projections, cell surface pitting and cell death and eventually exfoliation. This endothelial damage also occurred progressively as the temperature of incubation increased. Above 35.5°C, many areas of the endothelium began to show cell edge effects ("potato chip" appearance) which increased progressively at 37°C and 39°C, so that at 42°C and above, large areas of the endothelial surface were denuded and at 45°C and 50°C, the entire endothelial surface had no cells remaining. The effects seen at the endothelial surface generally could be characterized as somewhat more severe than those occurring at the epithelial surface (Table 2a).

By contrast, the epithelial changes observed at 39°C for various periods progressed in severity from normal at 0 time to almost normal at short times of incubation (with some cellular damage, and swelling in the peripheral but not central areas of the cornea), finally showing extensive swelling and some exfoliation of the epithelial surface after 45 minutes at 39°C in PBS.

TABLE 2A

Temperature:	35.5°	37°	39°	42°	45°	50°
Endothelium						
11/5/81	++++	ND	ND	ND	ND	ND
14/12/82	++++	++	++	o	oo	oo looks chewed
Epithelium						
11/5/81	++++	++++	++++	+++	+	o
14/12/82	++++	++++	+++ (border)	++	+	+
Stroma						
14/12/82	++++	++++ loose	+++ becoming more and more	++	++	+ disorganized

++++ normal
+++ few cells curled effected
++ big area affected
+ some denuded areas, most cells affected
o large denuded areas, most cells affected
oo all cells gone
ND Not done

TABLE 2B

Time:	0 Time	5 mins	10 mins	15 mins	30 mins	45 mins
11/5/81	++++ Ridges	++++	++++ 10% ridges	++++	++++	+
14/12/82	++++ Ridges	ND	++++	++++	+++ (see exp. 1)	ND

Further Studies of the Effects of UV irradiation On Corneas In Organ Culture

This work extends the studies of irradiation of corneas by ultraviolet light reported in our annual report, June 1982, DAMD17-82-C-2018. In previous studies, the corneas were incubated for 24 and 48 hr after irradiation, while in these experiments, the corneas were fixed immediately after exposure in Karnovsky's fixative, at 4°C and prepared for scanning electron microscopy. Controls (Fig. 4a showed smooth epithelial cell surfaces uniformly covered with normally appearing microvilli. After 15 sec. exposure to UV light (2 joules/m^2/sec) the cell surfaces appeared to have many small holes when compared to the controls. Cells could be divided into at least two types: most had normal appearance as expected for controls, while a smaller number (designated type b) had tiny microvilli or lacked them altogether. After 30 sec a larger number of type b cells was observed and some cells had begun to lift off, appearing like potato-chips. After 60 sec. still more areas consisting of type b cells was observed, and some limited areas were denuded of cells, giving an appearance of being "eaten away". After 120 sec large areas of epithelium were denuded of cells and almost all cells which were visible were curled up like potato chips and gave the appearance of being on the point of lifting off. Thus a progressive series of changes due to the UV-induced damage was observed.

In Vitro Tissue Culture of Cell from Cornea

This set of experiments was designed to reveal any special requirements for culturing of cell types in vitro. A brief summary of results follows:

1. Fibroblasts: Untransformed primary cells have been cultured, stored frozen and were found to have good viability when revived. Primary cells were transformed using a temperature-sensitive (Ts) Rous sarcoma virus: some of the transformed cells are being kept frozen as a reserve while cloning of the others (being carried by transfer) has begun.

2. Epithelium Cells: Untransformed primary cells have been cultured as reported in our annual report dated June 1982 for grant no. DAMD17-80-G-9480 and contract no. DAMD17-82-C-2018, and are being stored frozen with good viability when revived. Primary cells were transformed as described above with a Ts rous sarcoma virus: some of these transformed cells were stored frozen as a reserve while cloning of others (being carried by transfer) has begun.

3. Endothelial Cells: Culture of these cells has been more difficult to date, even though several attempts have been made to derive a cell line by transformation, no permanent line has been established.

RESULTS

In Vivo Irradiation with Millimeter Waves (Preliminary Results)

Extending the present techniques to millimeter wave studies may be somewhat simplified by the new technique of cryological-SEM-EDX; this technique is expected to have the following advantage:

(1) Because it is expected to be possible to monitor ionic concentrations in addition to observing the appearance by SEM of such sections, it should be convenient using the in vitro system to test parametrically several regimens. These might involve changes in irradiation conditions temperature, and medium supplementary to tests in vivo for which such variations are not easily possible.

It will be necessary to develop an appropriately modified chamber for the actual in vitro radiation, so that the following are satisfactory (1) control of temperature, flow rate and humidity of air in contact with upper corneal surface, (2) control of temperature and flow rate of circulating medium in contact with lower corneal surface, (3) transfer of the cornea on its grid to standard incubation medium after a period of irradiation. For in vivo irradiation modification of the current irradiation conditions to permit control of corneal surface temperatures in live animals by a flow of moist air on the corneal apical surface may be desirable.

(2) Damages Observed

Following irradiation with pulsed millimeter radiation, staining with either rose bengal or fluorescein was expected to reveal the types of damage detected by SEM in Figures 1, 2, and 3. Unfortunately although the damage could easily be detected by SEM, in rabbit eyes these two stains do not function ideally, a fact confirmed by Dr. A.T. Cullen, our consultant, when we asked him about this.

Epithelial Damage

The types of damage seen among epithelial cells closely paralleled those described for corneas damaged by heat treatment or UV:

In Vitro

Loss of raised cell borders, cell swelling, microvilli truncated or lost, with long processes extending at cell borders, which appeared irregular and jagged, eventual loss of many epithelial cells ensued leading to exposure of a disorganized

fibullar stromal surface. An intermediate stage involved the appearance visible by SEM of small areas of exposed stroma which should have been visible as points when stained with fluorescein.

Unfortunately these were not visible. The degree of damage seemed to be a function of the pulse peak power, when similar average power levels were compared. When times after irradiation were compared, the cell damage appeared to peak at about an hour and damage increased after that time as cells were lost by exfoliation. The thickness of fixed corneas increased from approximately 150 M to up to 350-600 M after 24 hr in some animals.

Exploration of parameters which may be involved in inducing corneal damage in vivo should include investigation of both effects of varying pulse parameters, of changing the rates of heating of the cornea for repeated short exposures, since this appeared to result in significant corneal damage when measurements of heating for SAR were done, even though the temperature did not increase over 43^{o}c during the runs.

Further measurements of SAR values should involve measurements of temperature elevation in both exposed and contralateral eyes, in order to be able to evaluate to what extent absorption of the mm waves will take place in the unexposed contralateral eye. If the hyperbolic (focussing) antenna is to be employed, it will be necessary to repeat all SAR measurements and to arrange for adequate shielding of one eye (to be used as control).

DISCUSSION

These results extend our previous studies of elevated temperature and nonionizing ultraviolet radiation on corneas in vitro using model systems in which damage observed will be a useful positive control to which the damage from microwave irradiation may be compared. The in vivo studies have indicated that millimeter wave-induced damage to the cornea which could be clearly seen by SEM was not easily detected in our hands by the techniques of fluorescein staining or rose Bengel staining. The observation of little damage in unexposed (contralaterial) eyes suggests that these are not significantly affected under conditions studied.

As noted in our 1982 Annual Report dated June 1982 and application observations of this staining, of aqueous flare and of corneal thickness using quantitative packymetry would have been facilitated by the availability of an ophthalmic microscope equipped to perform these measurements.

(1) Specific Absorption Rate (SAR)

Measurements of the SAR at the animal using average powers much higher than those used for the 20 min irradiation permitted rapid temperature increases to be measured under conditions which minimized heat loss by convection and conduction and thus gave as accurate a value for SAR as possible for the experimental conditions employed.

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Figure 1. (a) and (b) unirradiated (control) corneal epithelium at low and high magnification: Note smooth regular cell surface at low magnification and at high magnification, regular surface pattern of microvilli and raised borders between cells. (c) and (d) corneal epithelium at low and high magnification 24 hr following irradiation at low peak power condition (385.4 cm from antenna): Note potato-chip like curling of cell borders and swollen appearance of cells along with loss of microvilli leading to damaged cell surfaces (darker and light cells). (e) and (f) corneal epithelium at low and high magnificantion 24 hr following irradiation at high peak power condition (42.5) cm from antenna; Note prominent differences between "dark" and "light" cells at low magnification, granulation of cell surfaces and loss of microvilli, particularly apparent in dark cells and small pieces of cell debris attached to cell surfaces visible at higher magnification.

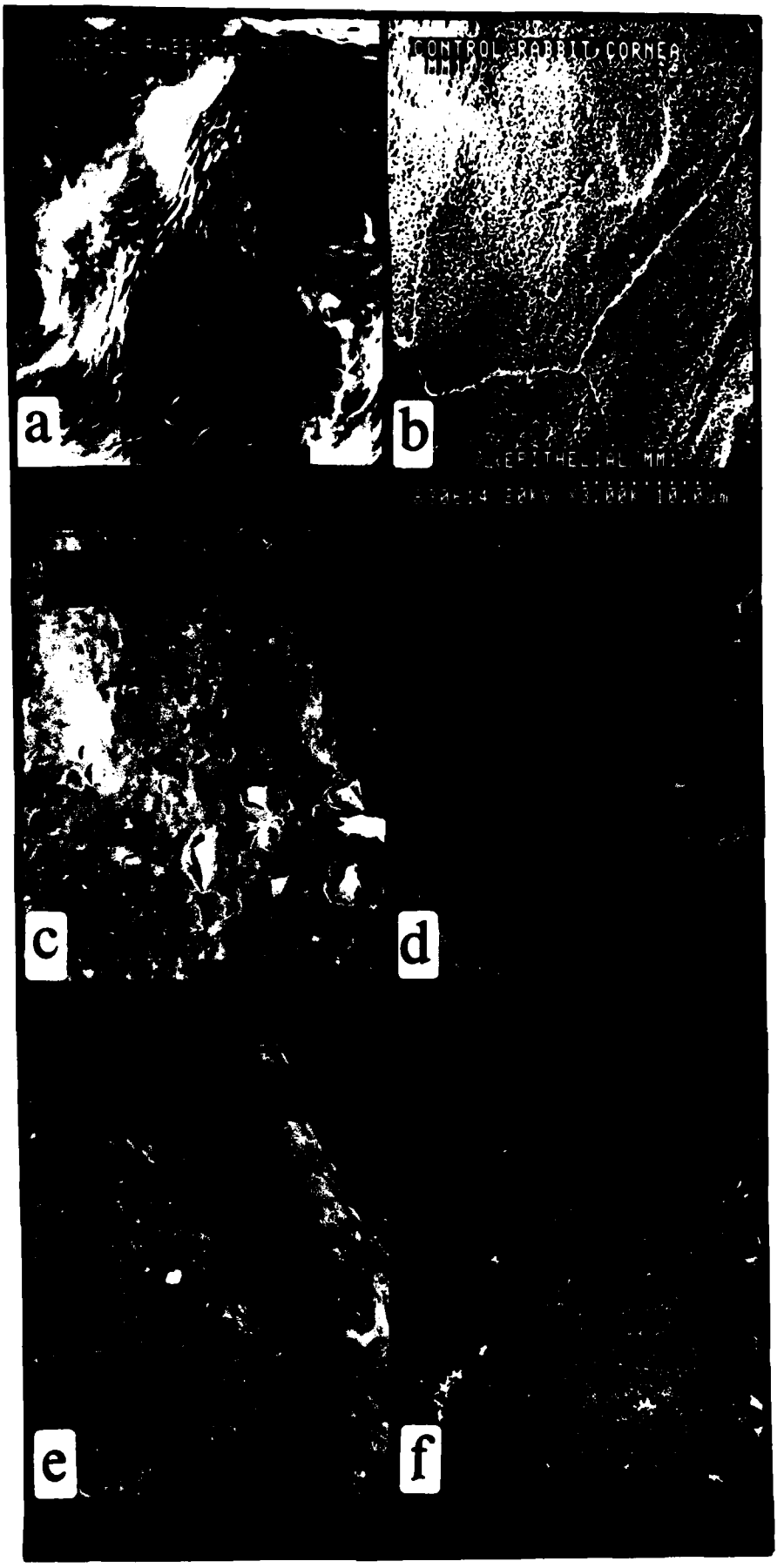

Figure 1: (a) and (b) unirradiated (control) corneal epithelium at low and high magnification.

Figure 2. (a) and (b) corneal epithelium of exposed eye 1 hr following irradiation at high peak power (42.5 cm from antenna). Note potato-chip-like curled edges of cells and dark and light cells at low magnification, and exposed stromal surface with interconnected collagen fibrils at high magnification. (c) and (d) corneal epithelium of exposed eye 1 hr following irradiation at high peak power (42.5 cm from antenna): Note swollen cells with granular surfaces and holes, and swollen nuclei and fibrous, degenerated cells with exposed cell cytoskeleton and fibrous underlying corneal stroma. (e) and (f) corneal epithelium of exposed eye 1 hr following corneal epithelium of exposed eye 1 hr following irradiation at high peak power (42.5 cm from antenna): Note exfoliation of cells down to single layer, and multilayered epithelium at lower magnification; at higher magnification and the cell surfaces show many small pock-marks and are granular and rough.

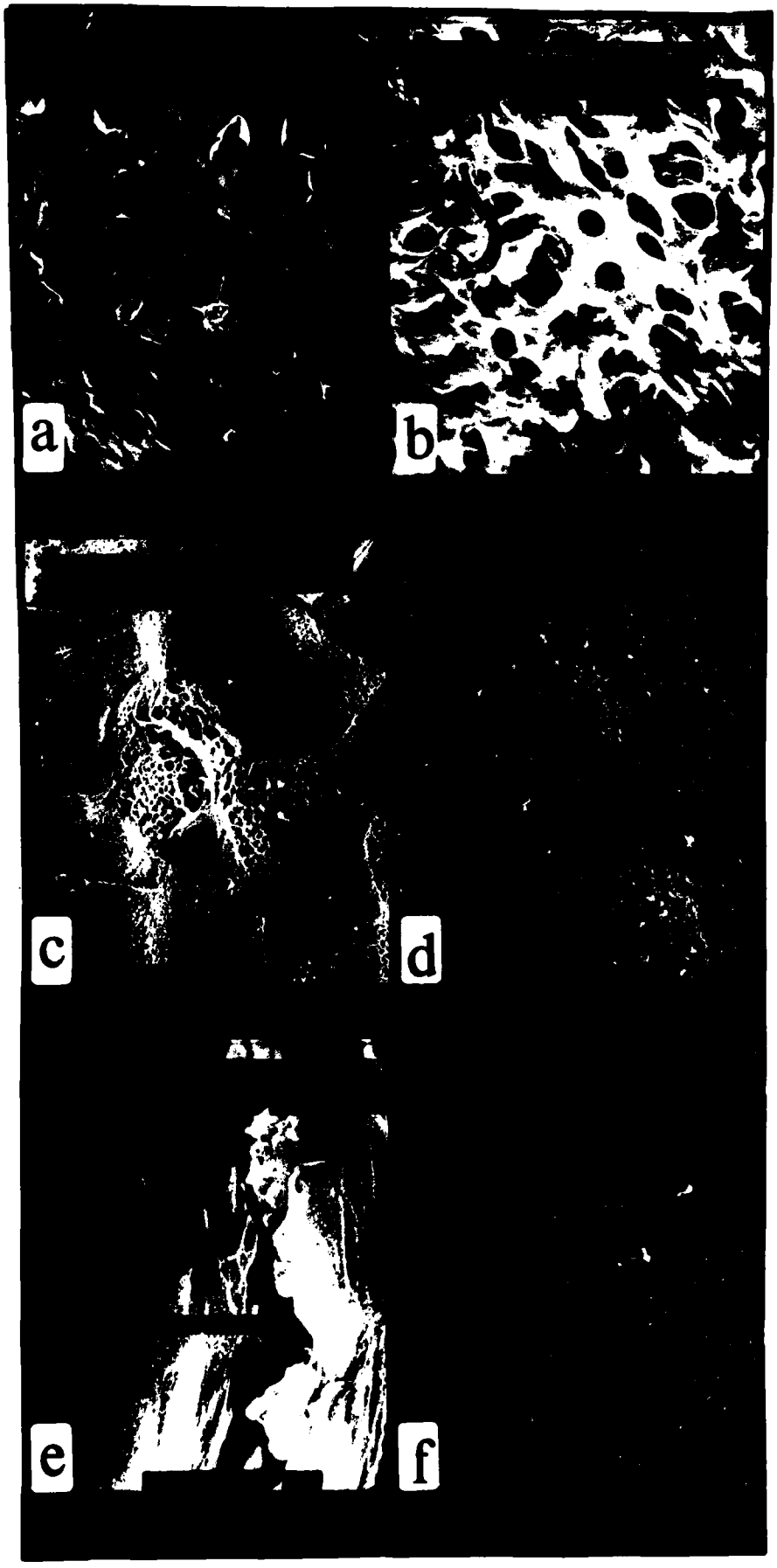

Figure 2: (a) and (b) corneal epithelium of exposed eye 1 hr following irradiation at high peak power (42.5 cm from antenna).

Figure 3. Scanning electron microscopy of corneal endothelial cells from rabbits exposed to pulsed millimeter waves at high and low pulse peak power and same average power. Exposure conditions were described in Figures 1 and 2, and rabbits were sacrificed either at 24 or 1 hr, or immediately after irradiation for the animals exposed so as to determine SAR. (a) endothelium of unexposed eye of rabbit (irradiated at low peak power setting): Note regular hexagonal cell shape, interdigilated cell boundaries and smooth cell surfaces with occasional microvilli. (b) corneal endothelium of exposed eye of rabbit at high peak power condition (42.5 cm from antenna) 1 hr after exposure: Note somewhat pitted irregular cell surface. (c) corneal endothelium of exposed eye of rabbit at low peak power condition (185.4 cm from antenna): Note pitted cell surface and small bits of surface debris. (d) and (e) corneal endothelial cell surface of exposed eye one hour and 24 hr after exposure to high peak power condition (42.5 cm from antenna) respectively: Note in (e) many pits and imperfections in cell surface and small processes protruding from surface. (f) corneal endothelial surface immediately following series of rapid temperature increases performed for determination of SAR. Note large area of complete exfoliation of endothelial cells, with dead cells and debris on the exposed corneal surface, dying cells (some fragmented)

at the edge of the remaining endothelial cells, and cell debris scattered on the exposed surface of the surviving endothelial cells.

Figure 3: Scanning electron microscopy of corneal endothelial cells from rabbits exposed to pulsed millimeter waves at high and low pulse power and same average power.

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